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LINEAGE ANALYSIS IN PULMONARY ARTERIAL HYPERTENSION

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14. ABSTRACT

Pulmonary arterial hypertension is characterized by inappropriate proliferation of neointimal cells that occlude the lumen of the microcirculation leading to right ventricular congestive failure and death. The neointimal cells express disorganized fibrils of smooth muscle actin. The origin of the neointimal cells remains unresolved: the neointima may arise from de-differentiation of vascular smooth muscle cells or from microvascular endothelial progenitor cells undergoing endothelial-to-mesenchymal transition. Aim 1 is to determine how endothelial to mesenchymal transition may contribute to neointimal vascular occlusion in pulmonary hypertension using genetic lineage marking in mice. Aim 2 is to characterize how Notch signaling regulates endothelial to mesenchymal transition. During the current funding period, successful Cre-lox genetic labeling of the endothelial lineage was achieved, and specificity of endothelial genetic lineage marking was confirmed by co-immunostaining of endothelial antigens, CD31 and VE-Cadherin. Successful inductiono of experimental pulmonary hypertension was achieved and demonstrated extensive contribution of endothelial genetic lineage-marked cells to neointimal vascular occlusion.

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INTRODUCTION:
Human Idiopathic Pulmonary Arterial Hypertension (IPAH) is characterized by neointimal vascular occlusion of the pulmonary microcirculation. Relentless elevations in pulmonary arterial pressures lead to death due to right ventricular failure (Lilienfeld and Rubin 2000, Rubin 1997). The pathology of PAH is characterized by abnormal expansions of neointimal cells expressing smooth muscle actin (Yi 2000).

There are few data on strategies that suppress neointimal formation being used to treat pulmonary hypertension (Gurubhagavatula and Palevsky 1997). The current medical management of PPH is directed at vasodilatation rather than the prevention of endothelial proliferation and neointimal formation. Prostacyclin may have beneficial effects on vascular remodeling, because some patients who do not demonstrate a vasodilator response to prostacyclin, appear to benefit from its use (Higenbottam 1993, Barst, 1996, Rich, 1999). A number of new agents, including simvastatin, hold the potential to attenuate disease progression (Kao and Faul 2003) (Kao 2005).

The pathogenesis of PAH involves 1) pulmonary vasoconstriction, 2) inappropriate proliferation of vascular cells in the intima and media, 3) inflammation and 4) thrombosis *in situ* (Fishman 1998, Mandegar 2004). All of these mechanisms may contribute to the development of PAH. The hypothesis of pulmonary vasoconstriction leading to medial hypertrophy and pulmonary hypertension was accepted for many years because of its intuitive similarity to the mechanism of development of systemic hypertension. Vasoconstriction associated with increased calcium influx contributes to smooth muscle hypertrophy in response to chronic hypoxia (Yu 1999, Mandegar, 2004).

Inappropriate hypertrophy and proliferation of cells within small pulmonary arterioles of patients with PAH is evident by analysis of the pathologic plexiform and concentric obliterative lesions that are characteristic of this disease. The lumens of small pulmonary arteries are diffusely narrowed by neointimal proliferation that consists of dedifferentiated vascular smooth muscle cells, myofibroblasts and endothelial cells (Tuder 1994, Veyssier-Belot, 1999). At the level of small pulmonary arteries, the occlusion by neointimal formation significantly exceeds muscularization of the medial component of the vessel wall.

Familial PPH occurs in about 10% of patients, and manifests an identical pathphysiology to sporadic PPH (Lee 1998, Yi, 2000). Recently, Deng et al. (Deng 2000) and Lane (Lane 2000) identified Bone Morphogenetic Protein Receptor Type II (BMPR2), located on the chromosome 2q33 as the genetic basis of familial PPH. Nearly 80% of patients with familial PAH have now been demonstrated to carry mutations in the BMPR2 gene. BMP receptors transduce antiproliferative signals to the nucleus through Smad proteins (ten Dijke 2003, Massague, 2000). Thus, familial PAH appears to arise from the loss of an antiproliferative signal or differentiating signal transmitted through the BMP signaling pathway. The important implications from this genetic discovery are that idiopathic PAH and anorexigen-induced PAH, may also arise from loss of antiproliferative signals. BMPR2 expression in the normal human lung is greatest in pulmonary endothelial cells, including microvascular ECs. Notably, lung specimens from patients with PPH and secondary PH showed marked attenuation of expression of BMPR2 in the pulmonary endothelium, with the greatest decreases observed in those patients who carried mutations in BMPR2 predicted to interfere with protein expression (Atkinson 2002).

The identity of the neointimal cells that occlude the lumens of small pulmonary arteries causing pulmonary hypertension remains a question of great significance. Based on the expression of smooth muscle actin (SMA), the neointimal cells have been traditionally considered to derive from the medial wall vascular smooth muscle cells, through a process of dedifferentiation. An alternative explanation was that the neointimal cells represented myofibroblasts that arose from differentiation of migrating adventitial fibroblasts (Arciniegas 2007). Neointimal cells that derived from the bone marrow were shown to incorporate into the wall of wire-injured systemic arteries, but no bone marrow-derived neointimal cells were observed in the pulmonary vascular lesions in monocrotaline-injected rats (Sahara 2007).

Endothelial to mesenchymal transition refers to the process in which a cell releases cell-to-cell contacts, loses polarity and undergoes remodeling of the cytoskeleton. Concurrent with the loss of endothelial antigens such as vWF, VE-Cadherin and PECAM, the cell will increase its expression of SMA and PDGF receptor (Arciniegas 2007). Arciniegas has been a pioneer in describing EnMT during normal development of the aorta and pulmonary artery in chick. The experiments are technically challenging because they depend on the ability to co-immunostain individual cells that are increasing SMA expression while decreasing expression of vWF or CD31. This lineage transition is a dynamic process and the experimental challenge is to capture the cells undergoing EnMT at the brief moment when there is simultaneous coexpression of different lineage markers.

Voelkel and Tuder described that plexiform lesions in human IPAH showed expression of the endothelial antigen vWF, and this discovery led them to propose that PAH represents a disease of monoclonal expansion of endothelial cells (Lee 1998). Other investigators and pathologists did not uniformly embrace this paradigm, because the vast majority of vascular lesions with neointima express SMA but no endothelial antigens. One way to reconcile Voelkel and Tuder's theory of PAH pathogenesis with the absence of endothelial antigens in the majority of neointimal cells, is to consider that neointimal cells may originally have been derived from endothelial progenitor cells that underwent endothelial to mesenchymal transition (Arciniegas 2007). In this proposal we aim to examine this question by using genetic lineage marking to permanently identify endothelial cells in the pulmonary microcirculation. Mice with endothelial cells permanently marked by expression of green fluorescent protein (GFP) reporter transgene will be subjected to our mouse model of pulmonary hypertension that produces neointimal lesions. If we detect GFP -labeled cells in the neointima, then we will have demonstrated unequivocally, that neointimal vascular occlusion in pulmonary hypertension can involve contributions from resident lung microvascular endothelial cells.

Endothelial to mesenchymal transitions have been shown to be strongly regulated by Notch signaling (Noseda 2006). Transduction of microvascular endothelial cells with activated Notch-1 intracellular domain (Notch-1 ICD) caused a dramatic change in morphology, new expression of SMA, fibronectin, PDGFR and substantial downregulation of expression of VE-cadherin, PECAM-1 and Tie-2. Here we propose to examine whether Notch-1 activation is detected in neointimal cells during the development of pulmonary hypertension. If we demonstrate that Notch-1 activation contributes to neointimal formation, we will test whether inhibitors of Notch activation, gamma secretase inhibitor, may suppress neointimal formation and pulmonary hypertension.

BODY:

Hypothesis: Pulmonary vascular injury triggers proliferation of lung microvascular endothelial progenitor cells capable of restoring the microvascular endothelium or undergoing endothelial to mesenchymal transition into smooth muscle actin-expressing neointimal cells that occlude the microcirculation, and regulation of this fate involves Notch-1 signaling.

Specific Aim 1: Determine how endothelial to mesenchymal transition may contribute to neointimal vascular occlusion in pulmonary hypertension using genetic lineage marking in mice. Mice with endothelial-specific expression of Cre recombinase (Tie-2 Cre, VE-Cadherin Cre) will be intercrossed with reporter mice (mT/mG double fluorescent Cre reporter) to permanently label cells of endothelial lineage. Subsequently, mice will undergo pneunomectomy followed one week later by intravenous injection of monocrotaline pyrrole. The fate of GFP-expressing cells of endothelial lineage will be correlated with immunofluorescent staining of endothelial markers CD31 and mesenchymal marker SMA. We demonstrate that GFP-expressing cells of endothelial lineage express SMA during development of pulmonary hypertension. The efficacy of simvastatin will be characterized to suppress EnMT and neointimal formation in experimental pulmonary hypertension.

Specific Aim 2: Characterize how Notch signaling regulates endothelial to mesenchymal transition. Cells active expressing Notch-1 intracellular domain (Notch1-ICD) will be detected by alpha-VLLS immunostaining. Expression of active Notch1IC will be correlated with cellular expression of endothelial and mesenchymal markers. Gamma secretase inhibitors of Notch activation will be evaluated for efficacy in suppressing EnMT, neointimal vascular occlusion and pulmonary hypertension in mice. We anticipate that inhibition of Notch signaling may represent a novel therapeutic approach to prevent and reverse pulmonary hypertension.

Preliminary Results:

Aim 1: We achieved endothelial genetic lineage marking by intercrossing VE-Cadherin Cre and Tie-2 Cre mice with mT/mG dual fluorescent Cre reporter mice. The reporter mice (developed by Liquin Luo lab at Stanford) express membrane-targeted tandem dimer Tomato (mT) fluorescent protein in all cells prior to Cre-mediated excision, and membrane-targeted green fluorescent protein (mG) after excision (Muzumdar, 2007). In the hierarchy of endothelial differentiation, Tie-2 is expressed early and VE-Cadherin is expressed as a late differentiation antigen of mature endothelial cells.

We developed protocols for mouse lung fixation (2% paraformaldehyde for 1 h) that preserved the endogenous fluorescence of the mT and mG proteins, and still allowed immunostaining for CD31, VE-Cadherin, SMA (smooth muscle alpha actin) or smooth muscle myosin heavy chain antigens. For these studies we were able to acquire a demo confocal microscopy system. A Leica DMI6000 inverted microscope with 40x and 63x oil immersion apochromatic objective lenses is coupled to a BD Carv II white light spinning disc confocal imager, with excitation and emission filters for 4 color acquisition and z-stacking. We acquire sequential 1 micron optical sections through physical cryosections of 100 micron thickness, and use a deconvolution algorithm for image enhancement followed by 3-D reconstruction through 10-15 microns using NIH Image J. Examining dual fluorescent labeled mice lungs combined with immunostaining, we have acquired remarkably informative images of pulmonary microvascular endothelial cells in alveolar capillaries and small pulmonary arteries.

As part of our control experiments to evaluate the Tie2 and VE-Cadherin Cre mT/mG endothelial genetic lineage marking, we performed immunostaining for CD31 and VE-Cadherin endothelial antigens. We observed good correlation between GFP-expressing endothelial genetic lineage-marked cells and CD31 and VE-Cadherin immunostaining.

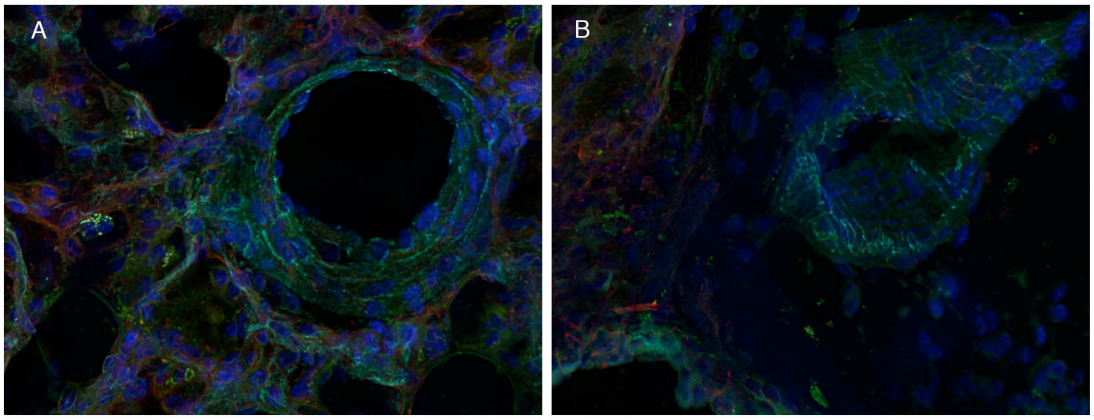

Figure 1. Endothelial genetic lineage marking correlates with endothelial antigen expression. Tie2 Cre x mT/mG excises dTomato and induces GFP expression in endothelial cells. **A.** CD31 (Cyan) **B.** VE-Cadherin (Cyan)

Interestingly, we discovered in normal lungs that a fraction of GFP-endothelial lineage-marked cells coexpressed SMA. We observed no expression of SMA by cells of endothelial genetic lineage in the aorta, heart, or kidney.

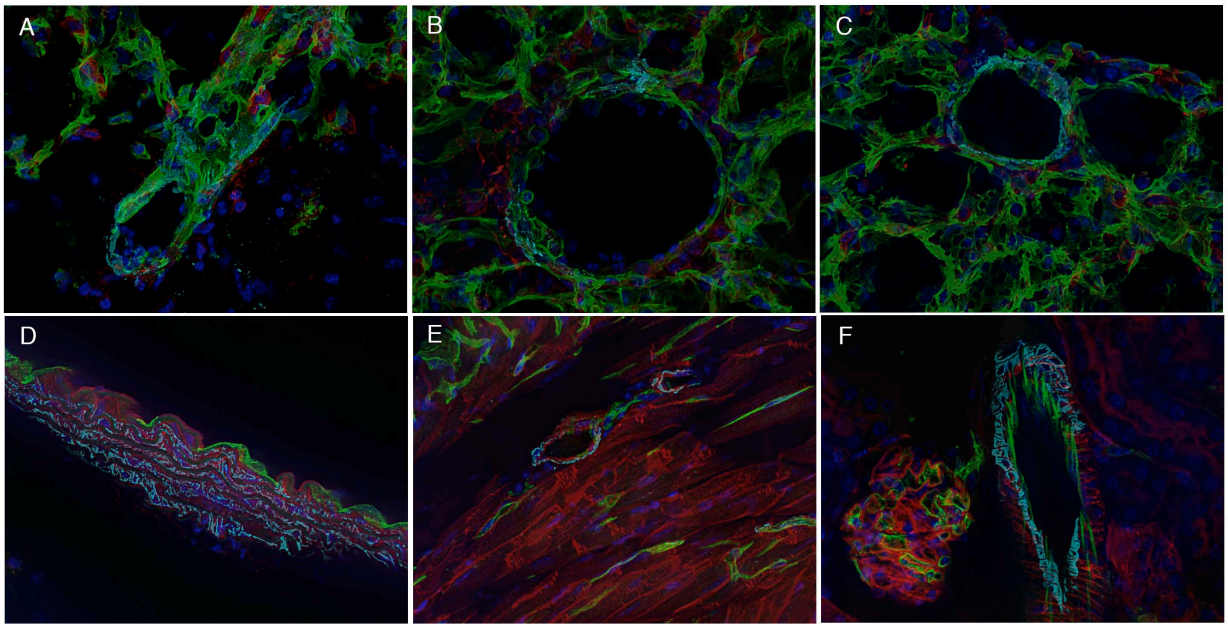

Figure 2. A subset of endothelial genetic-lineage marked cells express smooth muscle actin in pulmonary arteries but not in aorta, heart or kidney. VE-Cadherin Cre x mT/mG excises dTomato and induces GFP expression in differentiated endothelial cells. Cyan represents SMA immunostaining. **A-C.** Lung sections showing pulmonary vessels and alveolar capillaries **D.** Aorta **E.** Heart **F.** Kidney

Endothelial genetic lineage-marked mice were treated to develop experimental pulmonary hypertension. Mice underwent left pneumonectomy, and one week later the jugular vein was injected with 500 mcg of monocrotaline pyrrole in 25 microliters of dimethyl formamide. At day 35 after pneumonectomy, mice developed severe pulmonary hypertension with RVSP increased from 22 $\pm$ 3 mmHg to 54 $\pm$ 5 mmHg. Histology demonstrated development of neointimal vascular occlusion in small pulmonary arteries that correlated with experimental pulmonary hypertension.

Cells contributing to the neointimal vascular occlusion of pulmonary arteries expressed GFP, indicating endothelial genetic lineage origin. Neointimal cells demonstrated prominent expression of SMA, colocalizing with GFP expression in cells of endothelial genetic lineage.

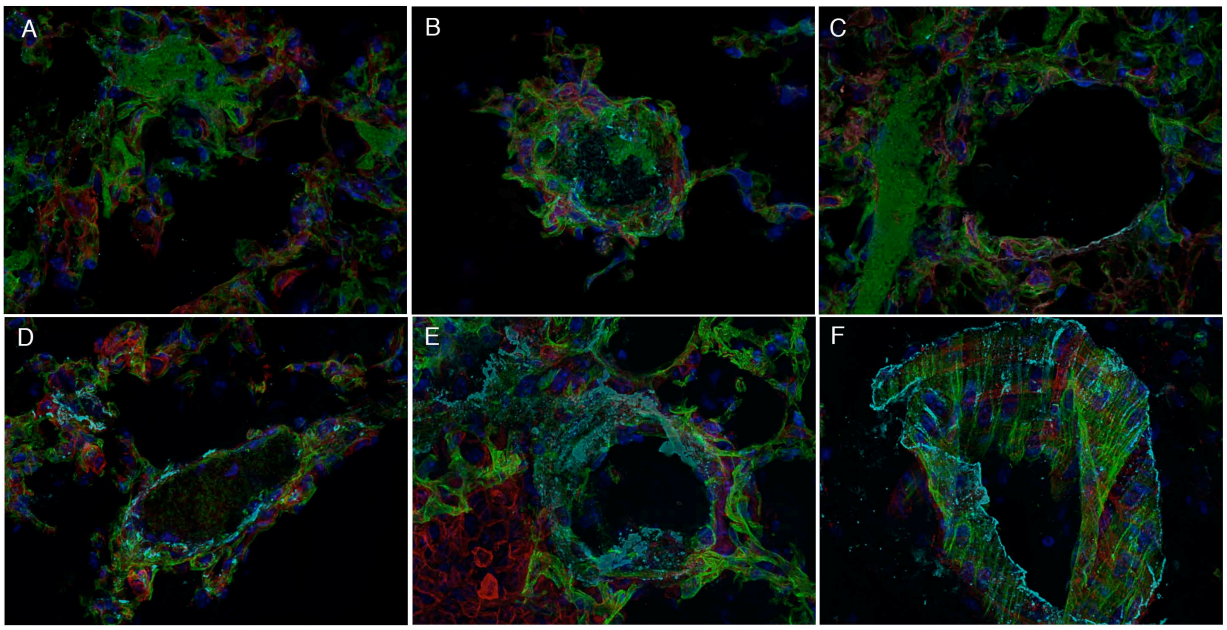

Figure 3. Neointimal vascular occlusion in experimental pulmonary hypertension involves cells of endothelial genetic lineage expressing smooth muscle actin. A-C. Tie2 Cre x mT/mG induces GFP expression in endothelial genetic lineage. Vascular occlusion exclusively by GFP-marked cells with coexpression of SMA (cyan). **D-F.** VE Cad Cre x mT/mG induces GFP expression in endothelial lineage. Vascular occlusion predominantly by GFP-marked cells coexpressing SMA (cyan).

These data prove that neointimal cells contributing to vascular occlusion in experimental pulmonary hypertension originate from endothelial genetic lineage, and coexpress smooth muscle actin.

Aim 2: We have not yet embarked on experiments addressing this aim. We will perform immunostaining for activated Notch ICD and determine if there is colocalization with neointimal cells of endothelial genetic lineage. If we demonstrate Notch signaling activation in neointimal cells in our model of experimental pulmonary hypertension, then we will proceed to evaluate the effects of Notch signaling inhibitors on the development of pulmonary hypertension.

KEY RESEARCH ACCOMPLISHMENTS:

1) Successful Cre-lox labeling of endothelial genetic lineage in mouse lung, by intercrossing Tie-2 Cre and VE-Cadherin Cre endothelial driver mice with mT/mG dual fluorescent switch reporter mice. Microvascular pulmonary endothelial cells express GFP (green) while non-endothelial cells express dTomato (red).

2) Successful immunostaining for endothelial antigens CD31 and VE-Cadherin in genetic lineage marked mice confirms endothelial lineage marking is specific and complete.

3) Immunostaining for smooth muscle actin in endothelial lineage-marked mice reveals that a subset of endothelial cells coexpress SMA in non-injured lungs. Endothelial cells in heart, kidney and skeletal muscle do not express SMA.

4) Neointimal cells contributing to vascular occlusion in experimental pulmonary hypertension originate from endothelial genetic lineage, and coexpress SMA.

REPORTABLE OUTCOMES: Manuscript in preparation

CONCLUSION: Our results demonstrate that the endothelial genetic lineage contributes to neointimal vascular occlusion in experimental pulmonary hypertension. This discovery shall focus future investigations upon novel therapies that suppress endothelial to mesenchymal transition and inappropriate expansion of pulmonary microvascular endothelial cells after injury.

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